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Molecular and Genetic Analysis of Condensin Function in Vertebrate Cells

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We engineered mutants into residues of SMC2 to dissect the role of ATPase function in the condensin complex. These residues are predicted to be involved in ATP binding or hydrolysis and in the Q-loop, which is thought to act as a mediator of conformational changes induced by substrate binding. All the engineered ATPase mutations resulted in lethality when introduced into SMC2 null cells. We found that ATP binding, but not hydrolysis, is essential to allow stable condensin association with chromosomes. How SMC proteins bind and interact with DNA is still a major question. Cohesin may form a ring structure that topologically encircles DNA. We examined whether condensin behaves in an analogous way to its cohesin counterpart, and we have generated a cleavable form of biologically active condensin with PreScission protease sites engineered into the SMC2 protein. This has allowed us to demonstrate that topological integrity of the SMC2-SMC4 heterodimer is not necessary for the stability of the condensin complex *in vitro* or for its stable association with mitotic chromosomes. Thus, despite their similar molecular organization, condensin and cohesin exhibit fundamental differences in their structure and function.

INTRODUCTION

The assembly of DNA into condensed chromosomes during mitosis is essential for the faithful segregation of the genome into daughter nuclei. Several studies have shown that the condensin complex has a crucial role in the formation of structurally stable mitotic chromosomes and their segregation *in vivo* (Strunnikov *et al.*, 1995; Hagstrom *et al.*, 2002; Hudson *et al.*, 2003; Hirano, 2005). Although vertebrate chromosomes lacking condensin manage to compact their chromatin almost normally, they lose their organized architecture during anaphase as Repo-Man targets protein phosphatase 1 to the separating chromatids (Vagnarelli *et al.*, 2006). Thus, one role of the condensin complex is to direct and/or regulate the association of other nonhistone proteins with mitotic chromosomes. Although the requirement for condensin function in chromosome architecture is well established, its mechanism of action remains an open question.

The two forms of condensin, condensin I and II, are pentameric complexes composed of the SMC2 and SMC4 ATPases plus three auxiliary subunits (CapG/G2, CapD2/D3, and CapH/H2) (Ono *et al.*, 2003; Hirota *et al.*, 2004). The two SMC subunits are known to be responsible for ATPase activity, which is essential for condensin function (Stray and

Lindsley, 2003). However it is not known how the ATPase activity contributes to chromosome condensation.

Condensin subunit ScII/SMC2, first isolated as a component of the chromosome scaffold fraction (Saitoh *et al.*, 1994), belongs to the structural maintenance of chromosome (SMC) family, a large family of chromosomal ATPases involved in chromosome condensation, sister chromatid cohesion, and DNA repair (Cobbe and Heck, 2000; Hirano, 2006). These proteins share aspects of common architecture with ATP-binding cassette (ABC) membrane transporters (Saitoh *et al.*, 1995; Hopfner *et al.*, 2001). Both contain Walker A and B consensus sequences as well as the highly conserved 'LS-GGQ' signature sequence or C-motif. In DNA mismatch repair, the MutS ABC ATPase uses ATP binding to recognize and bind misrepaired DNA (Junop *et al.*, 2001). In double-stranded break repair, Rad50 uses ATP to bind and bridge DNA double-strand breaks (Chen *et al.*, 2001; Lobachev *et al.*, 2002), and ABC transporters use the energy from ATP hydrolysis to transport substances across membranes (Hyde *et al.*, 1990).

A common theme has emerged among ABC-like ATPases, despite the wide range of functions carried out by this diverse protein family. ATP binding through the signature and Q-loop motifs causes conformational changes necessary to accomplish functions as diverse as DNA repair and transmembrane transport (Hopfner and Tainer, 2003). Although several studies indicate that ATPase activity of condensin is needed for its enzymatic properties, the precise role of ATP binding and hydrolysis is not yet known. *In vitro* studies in *Xenopus* egg extracts show that positive knotting and super-

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coiling of plasmid DNA by high concentrations of condensin are ATP dependent (Kimura and Hirano, 1997; Kimura *et al.*, 1998), and studies using nano-manipulation of a single DNA molecule show that condensin I can compact DNA in an ATP hydrolysis-dependent manner (Bazett-Jones *et al.*, 2002).

Here, we report the *in vivo* analysis of condensin ATPase function by using a systematic mutagenesis of all known SMC2 ATPase domains, including the Q-loop, signature motif, and Walker A and B motifs. We have also generated the first biologically active cleavable form of SMC2, allowing us to break the putative condensin complex ring *in vitro* and determine whether condensin might function in an analogous way to cohesin by topologically embracing DNA (Gruber *et al.*, 2003; Ivanov and Nasmyth, 2005). These studies were performed in DT40 cells with a conditional knockout of the SMC2 gene, so that all phenotypes observed reflect the activity of a homogeneous mutant complex, with no background of the wild-type protein.

Our results reveal several fundamental and intriguing differences between the condensin and cohesin complexes. We demonstrate that SMC2 ATP binding, but not hydrolysis, is required for condensin to stably associate with mitotic chromosomes. However, ATP binding is not required for formation of the condensin complex. We also show that disruption of the putative condensin ring does not affect the integrity of the complex or its ability to associate with mitotic chromosomes.

MATERIALS AND METHODS

Cell Culture and Transfections

The chicken DT40 cell culture and repression experiments in the SMC2 conditional-knockout cell line were performed as described previously (Hudson *et al.*, 2003; Vagnarelli *et al.*, 2006). For transient transfections (Figure 3A), 1.0×10^7 DT40 cells were rinsed with phosphate-buffered saline (PBS) and then suspended into 0.1 ml of nucleofector solution V per cuvette. DNA (2–10 μ g) was added to each cuvette and electroporated (Program B-30, Nucleofector II; Amaxa, Cologne, Germany).

Construction of SMC2 ATPase Mutants and PreScission SMC2

Wild-type SMC2 cDNA, SMC2 PreScission, and SMC2 ATPase mutants were cloned downstream of the 3822 base pairs SMC2 promoter fragment. Point mutations to generate ATPase mutations were introduced into the SMC2 cDNA by using a QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by sequencing. The 45-amino acid streptavidin binding peptide (Keefe *et al.*, 2001) for experiments in Figures 2 and 3 was linked to the C terminus of SMC2 by using a unique SalI site engineered over the stop codon. The TrAP tag for the PreScission SMC2 construct was also inserted at the SalI site. To generate SMC2 PreScission, oligonucleotides containing the eight-amino acid (Leu Glu Val Leu Phe Gln Gly Pro) PreScission protease recognition sites were cloned into SMC2 at amino acid position 386 and 949 by using a unique AvrII site engineered into the SMC2 cDNA with QuikChange XL under the control of the SMC2 3.8-kb promoter fragment. Oligonucleotide sequences used for mutagenesis, insertion of PreScission sites, streptavidin binding peptide (SBP), TrAP tags, and cloning of SMC2 promoter are described in Supplemental Data.

Condensin Affinity Purification

SBP-tagged or SBP/His/S-tagged condensin was isolated from DT40 cells by using streptavidin beads (Chemical Pierce, Rockford, IL) for the SBP tag and S-protein agarose beads (Novagen, Darmstadt, Germany) for the S tag. In general, 10^8 colcemid blocked cells were frozen as cell pellets and stored at -80°C . Lysis was performed on thawed cell pellets by using 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% NP-40, 1 mM CaCl_2 , 30 $\mu\text{g}/\text{ml}$ RNase A, 40 $\mu\text{g}/\text{ml}$ micrococcal nuclease, 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ each chymostatin, leupeptin, antipain, and pepstatin A, and 1 mM phenylmethylsulfonyl fluoride for 45 min on ice followed by the addition of EDTA and deoxycholate to 1 mM and 0.1%, respectively. Lysates were centrifuged at 4°C for 10 min at 14,000 rpm. Streptavidin agarose beads (1 ml) were mixed with cleared lysate (supernatant) for 2 h, rotating at 4°C in a final volume of 10 ml. Beads were washed in wash buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, or 250 mM for PreScission assays, 0.5% NP-40) three times and eluted in elution buffer (50

mM Tris-HCl, pH 7.4, 50 mM NaCl for Figure 2, C and D (or 250 mM NaCl for Figure 5), 0.5% NP-40, 0.1% deoxycholate, and 4 mM biotin). For Figure 2C, the sample was eluted from streptavidin beads by boiling in SDS sample buffer. For double affinity preparations (Figure 5, I and J), the biotin eluate was then added to 700 μl of S-protein agarose beads. Subsequent binding and washing conditions were as for streptavidin beads with sample eluted from S-beads by boiling in SDS sample buffer.

Preparation of Mitotic Chromosome and Scaffolds

DT40 SMC2^{ON/OFF} cells at densities of $0.8\text{--}1.0 \times 10^6/\text{ml}$ were incubated with 0.5 $\mu\text{g}/\text{ml}$ nocodazole for 12 h, resulting in a mitotic index of up to 80%. Mitotic chromosomes were isolated in polyamine-EDTA buffers as described previously (Lewis and Laemmli, 1982), except that the detergents used after cell lysis were 0.1% Ammonyx Lo or 0.1% *n*-dodecyl- β -maltoide instead of digitonin. Chromosomes used for immunofluorescence (Figure 6G) were purified up to the glycerol gradient step. Typically, 2–5 OD₂₆₀ units were obtained from 500 ml of cultured cells. For chromosome scaffold preparation, isolated chromosomes were incubated in 0.5 mM CaCl_2 and 40 $\mu\text{g}/\text{ml}$ micrococcal nuclease for 20 min on ice. CuSO_4 (0.5 mM) was then added under nitrogen gas and incubated for 10 min on ice. For histone depletion, the chromosomes were incubated in 2 M NaCl for 20 min (Lewis and Laemmli, 1982). The insoluble scaffold fraction was pelleted at $6800 \times g$ and solubilized in SDS sample buffer.

PreScission Protease Digestion

Isolated mitotic chromosomes were treated with PreScission protease for 16 h at 4°C with 80 μl of enzyme (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), and scaffolds were isolated as described. For pull-down and digestion assays (Figure 5), condensin bound to streptavidin beads was washed a further two times in PreScission buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.01% Triton, and 1 mM EDTA) and suspended in 1-ml final volume of the same buffer. Digestion was performed for 16 h on a rotating platform at 4°C with 80 μl of enzyme.

Immunoblotting Analysis

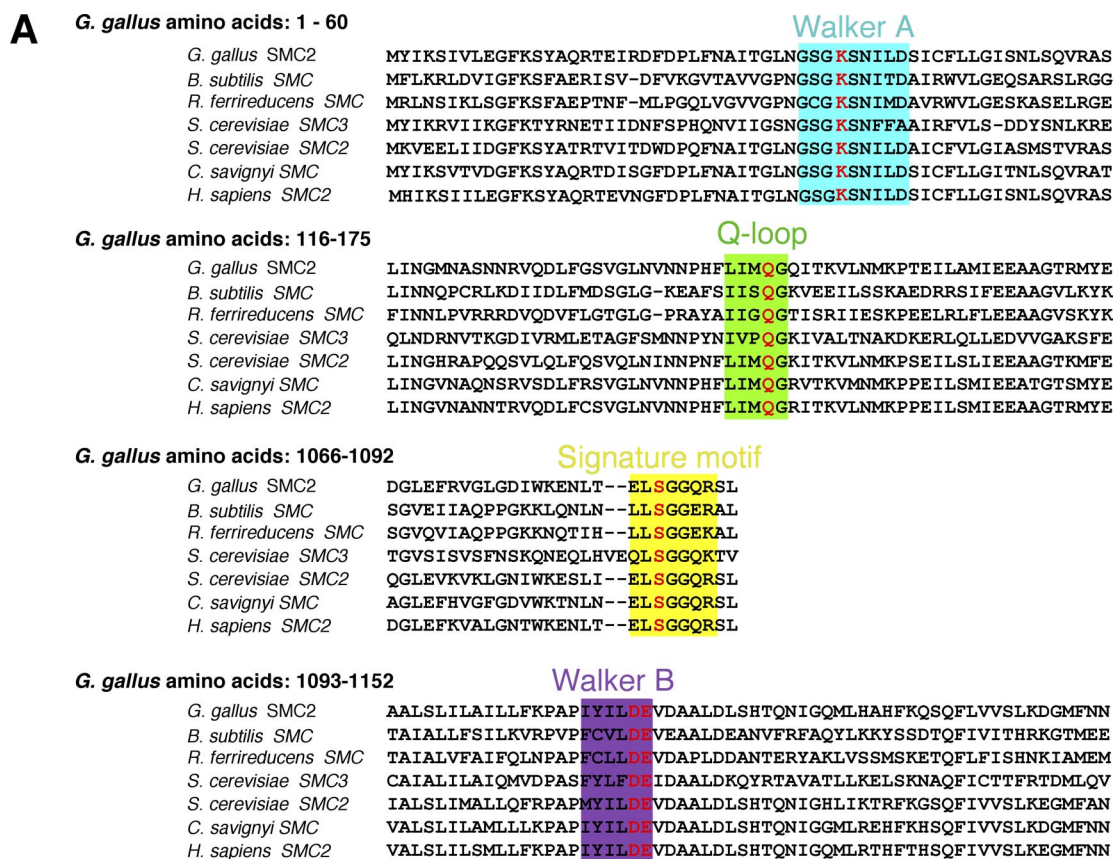
Protein samples for total lysate, affinity-purified condensin, and from isolated chromosomes were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto nitrocellulose membrane (GE Healthcare). After blotting, the membranes were stained with Ponceau S (Sigma-Aldrich, St. Louis, MO). Membranes were blocked with 5% skimmed milk in PBS and processed for enhanced chemiluminescence by standard methods. Antibodies used were as follows: rabbit anti-KIF4A at 1:500, mouse anti-tubulin B512 (Sigma-Aldrich) at 1:1000, anti-SMC4 (Acris Antibodies, Hiddenhausen, Germany) at 1:500, anti-ScII M, N (Saitoh *et al.*, 1994) at 1:1000, rabbit anti-CAP-H (Vagnarelli *et al.*, 2006) at 1:1000, rabbit anti-topoisomerase (Topo) II α (Hoffmann *et al.*, 1989) at 1:1000, and monoclonal anti-SBP (1:300). The *Gallus gallus* CAP-D2 antibody was raised to a 28-kDa peptide fragment in rabbit, corresponding to amino acids 1077–1324, and it was used at 1:1000.

Quantification of Antigen Recovery in the Scaffold and Supernatant Fractions

Scaffold and supernatant samples were prepared as described above and boiled in SDS-sample buffer. A range of volumes of these samples was then subjected to SDS-PAGE in 7.5% polyacrylamide gels, and immunoblotting as described above. The intensities corresponding to each antigen were quantified using ImageJ (NIH Image; <http://rsb.info.nih.gov/ij/>). To calculate the protein recovery in the scaffold and supernatant fractions, standard curves were made from at least four points corresponding to different loading volumes. Experimental values were then extrapolated from the linear portion of these curves. The percentage of each protein recovered in the scaffold fraction was calculated as follows: [scaffold/(scaffold + supernatant)] $\times 100$.

Indirect Immunofluorescence Microscopy

Chromosome spreads were prepared from cells either dropped onto slides (Figure 2B and Supplemental Figure S3) or grown on concanavalin A coverslips (Figures 3A and 4A) and processed as follows. Cultured cells were blocked in mitosis with colcemid (100 ng/ml) for 2 h, hypotonically swollen in 75 mM KCl for 5 min, and fixed with cold methanol:acetic acid (-20°C) (3:1). Primary antibodies (anti-KIF4A at 1:500, SMC2 M at 1:200, CAP-D2 at 1:200, CAP-H 1:200, and SBP at 1:50) in TEEN buffer (1 mM triethanolamine-HCl, pH 8.5, 0.2 mM Na-EDTA, and 25 mM NaCl) with 0.1% Triton and 1% bovine serum albumin (BSA) were incubated for 30 min. Cells were washed three times in KB buffer (10 mM Tris-HCl, pH 7.7, 150 mM NaCl, and 0.1% BSA), and fluorescence-labeled secondary antibodies were applied (Invitrogen, Carlsbad, CA; for Figure 4 at 1:500; Jackson ImmunoResearch Laboratories, West Grove, PA; Figures 2B and 3A and Supplemental Figure 2 at 1:200) and counterstained with 4,6-diamidino-2-phenylindole (Calbiochem, Darmstadt, Germany). Three-dimensional data sets were collected with a DeltaVision system (Applied Precision, Issaquah, WA) based on an IX-70 inverted microscope (Olympus, Tokyo, Japan) with a Sedat filter set (Chroma Tech-



B

Mutation	SMC2 Domain	Predicted effect	Prosite reference
D1113A	Walker B	No ATP binding	PS50100
E1114Q	Walker B	Slows ATP hydrolysis	PS50100
K38I	Walker A	No ATP binding	PS00017
S1086R	Signature motif	No ATP dependent dimerization	PS00211
Q147L	Q-loop	No water and Mg ⁺⁺ ion binding	

Figure 1. ATP binding and hydrolysis are required for SMC2 function. (A) Sequence alignment using MUSCLE (Edgar, 2004) of conserved SMC ATPase domains from *G. gallus* SMC2 (NP_990561.1), *B. subtilis* SMC (NP_389476.1), *Rhodospirillum rubrum* SMC (ABD69923.1), *Saccharomyces cerevisiae* SMC3 (NP_012461.1), *S. cerevisiae* SMC2 (NP_116687.1), *Ciona savignyi* SMC (ENSCAVP00000005455), and *Homo sapiens* SMC2 (NP_006435.2). Site-directed mutagenesis positions are highlighted in red. (B) Mutations engineered into SMC2 and their expected effects on ATPase activity.

nology, Brattleboro, VT) driven by the SoftWoRx software under standard conditions. All the image files were captured as raw (r3d) files and deconvolved (d3d) files subsequently prepared by the SoftWoRx software (Applied Precision) deconvolution algorithm. Three-dimensional data sets were converted to Quick Projections in SoftWoRx and then imported into Adobe Photoshop (Adobe Systems, Mountain View, CA) for final presentation. Levels were adjusted across each entire image to lower nonspecific background haze using the standard Photoshop adjustment of levels.

RESULTS

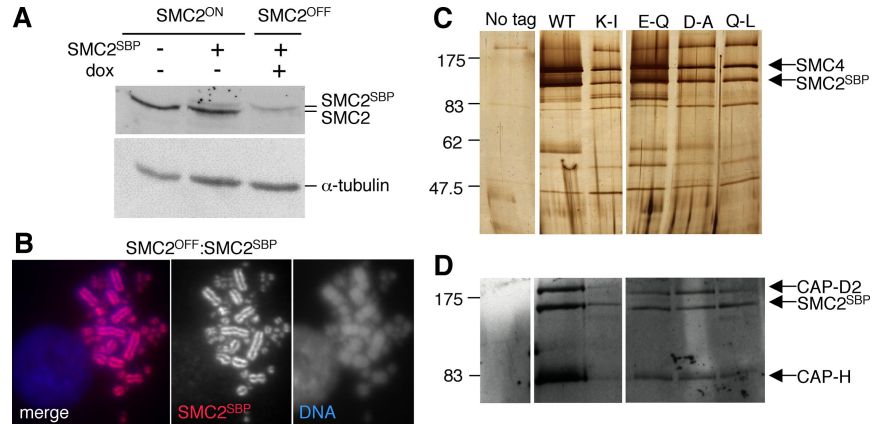
ATP Binding and Hydrolysis Are Required for SMC2 Function

We introduced mutations into four conserved domains of SMC2 implicated in ATPase activity (Walker A, Q-loop, Signature motif, and Walker B) (Figure 1, A and B). All

mutants were expressed in SMC2^{ON/OFF} conditional knock-out cells under control of a promoter fragment consisting of the 3.8-kb genomic fragment directly upstream of the start codon of SMC2. This allowed the phenotype of the mutant proteins to be analyzed in the absence of endogenous SMC2. Wild-type SMC2 expression driven by this promoter fragment complemented SMC2^{OFF} cells (Supplemental Figure S1, A and B).

Our mutagenesis strategy was based on the premise that the mutations would be expected to have the same effect in SMC2 as they do in bacterial SMC proteins (Hirano *et al.*, 2001; Hirano and Hirano, 2006), cohesin SMCs (Arumugam *et al.*, 2003; Weitzer *et al.*, 2003; Arumugam *et al.*, 2006) ABC transporters and RAD 50 (Hopfner *et al.*, 2000; also see

Figure 2. SMC2 ATPase activity is not essential for the formation of the condensin I holo complex. (A) SBP-tagged SMC2 is able to rescue SMC2^{OFF} cells. SMC2 conditional knockout cells (5×10^5 cells), SMC2^{ON}SMC2^{SBP} and SMC2^{OFF}SMC2^{SBP} were resolved by 10% SDS-PAGE and immunoblotted with anti-SMC2 (ScII-M). Anti-tubulin was used as a loading control. (B) Chromosome structure and condensin localization are normal for cells expressing only SMC2^{SBP}. Chromosome spreads for SMC2^{OFF}SMC2^{SBP} cells were immunostained with anti-SMC2 (ScII-M) antibody. (C) Condensin purification using a single pull-down with the SBP tag. Silver stained gel of SMC2^{OFF}SMC2^{SBP} cells purified over streptavidin beads and eluted with biotin. Condensin complexes were isolated from cells expressing SMC2^{SBP}-tagged WT, SMC2^{SBP} ATPase mutants (E1114Q, K38I, D1113A, and Q147L) and untagged (no tag) and visualized by silver staining. Equivalent amount of cells (10^6) were added for each lane. (D) Immunoblot analysis of corresponding samples from C by using antibodies for the condensin I subunits CAP-H and CAP-D2 show there is no significant difference between ATPase mutants and WT cells.



summary in Figure 1B). A Walker A box mutation, K38I, and the D1113A mutation in the Walker B motif are predicted to prevent ATP binding, whereas an E1114Q "transition state" mutation in the Walker B box would slow the rate of ATP hydrolysis that is thought to be required for head disengagement in SMC complexes (Hirano, 2006). The S1086R mutation in the C-terminal signature motif is predicted to allow ATP binding but prevent ATP hydrolysis. These four residues are completely conserved among SMCs and ABC transporters and have been extensively characterized (Hirano *et al.*, 2001; Arumugam *et al.*, 2003, 2006; Weitzer *et al.*, 2003; Hirano and Hirano, 2006), but little is known of their role in the condensin complex *in vivo*. Mutation of the conserved Q-loop (Q147L) is predicted to block binding of the attacking nucleophile (water) and Mg^{2+} ions involved in ATP hydrolysis (Hopfner *et al.*, 2000). It has been suggested that contacts between the Q-loop glutamine, and the active site magnesium and nucleophile make it a good candidate for the transmission of allosteric changes caused by substrate binding (Hopfner and Tainer, 2003). However, no SMC Q-loop mutant has been characterized *in vivo* in detail to date.

SMC2^{ON/OFF} cell lines stably expressing the above-mentioned mutants were isolated for further analysis. Clones were selected on the basis that they expressed levels of SMC2 cDNA similar to those in wild-type DT40 cells as determined using semiquantitative polymerase chain reaction (PCR) (data not shown). SMC2 mutants were untagged to exclude the low possibility that the tag might produce an additive affect in combination with the mutation (note that tagged wild-type SMC2 fully rescues SMC2 function). In multiple transfections of constructs expressing the various SMC2 ATPase mutations, no clones were isolated that were able to rescue life in SMC2^{OFF} cells. Instead, all resulting cell lines gave mitotic phenotypes similar to those of SMC2^{OFF} cells when scored for anaphase and telophase chromosome bridges, mitotic index, multipolar spindles, and cytokinesis bridges (Supplemental Figure S1, C–F). Thus, all residues mutagenized are essential for SMC2 function.

SMC2 ATPase Activity Is Not Required for Formation of the Condensin I Holo complex

To analyze the effect of the above mutations on condensin complex assembly, SMC2 wild-type and mutant constructs

were tagged with the 45-amino acid SBP, and stable cell lines were generated containing mutant or wild-type SMC2^{SBP} in the conditional SMC2^{ON/OFF} background. SBP-tagged wild-type SMC2 expressed from the endogenous SMC2 promoter complemented SMC2 null cells and immunoblotting analysis revealed only SMC2^{SBP} protein after doxycycline was added (Figure 2A). Chromosomes containing only SMC2^{SBP} had normal morphology and displayed the characteristic SMC2 axial staining (Figure 2B). Thus, SMC2^{SBP} seems to be a fully functional protein.

To assess whether ATPase mutations of SMC2 affect formation of the condensin complex, pull-downs were performed from stable cell lines expressing the wild type (WT), K38I, D1113A, E1114Q and Q147L ATPase mutants tagged with SBP. Proteins from equivalent amounts of cells were silver stained (Figure 2C) or immunoblotted for the condensin I components CAP-D2 and CAP-H (Figure 2D). Silver stain analysis revealed that SMC2-SMC4 heterodimer formation was not affected by any of the mutations, and immunoblotting with CAP-D2 and CAP-H antibodies indicated that the non-SMC subunits of condensin I assembled normally with all of the various mutants. This agrees with *in vitro* data using baculovirus expressed condensin I (Onn *et al.*, 2007). Thus, a functional condensin ATPase is not a prerequisite for assembly of the complex *in vivo*.

SMC2 ATP Binding but Not Hydrolysis Is Essential for Loading Condensin onto Mitotic Chromosomes

Given that the condensin complex formed, one explanation for the failure of SMC2 ATPase mutations to rescue condensin function might be that ATP binding and/or hydrolysis is required for the complex to assemble onto mitotic chromosomes. To determine whether the various mutant condensin complexes could still load onto mitotic chromosomes, SMC2^{ON/OFF} cells were transiently transfected with vectors expressing wild-type or mutant SMC2 10 h after addition of doxycycline to turn off the conditional wild-type allele. Transfected cells were allowed to express the various SMC2 mutants for 16 h, hypotonically swollen, and then fixed with methanol:acetic acid (3:1) and stained with anti-SBP.

Mutations affecting ATP binding (D1113A, K38I) as well as the Q-loop mutant (Q147L) prevented any detectable loading of condensin onto mitotic chromosomes. Instead, a diffuse cytoplasmic staining was observed. In contrast, con-

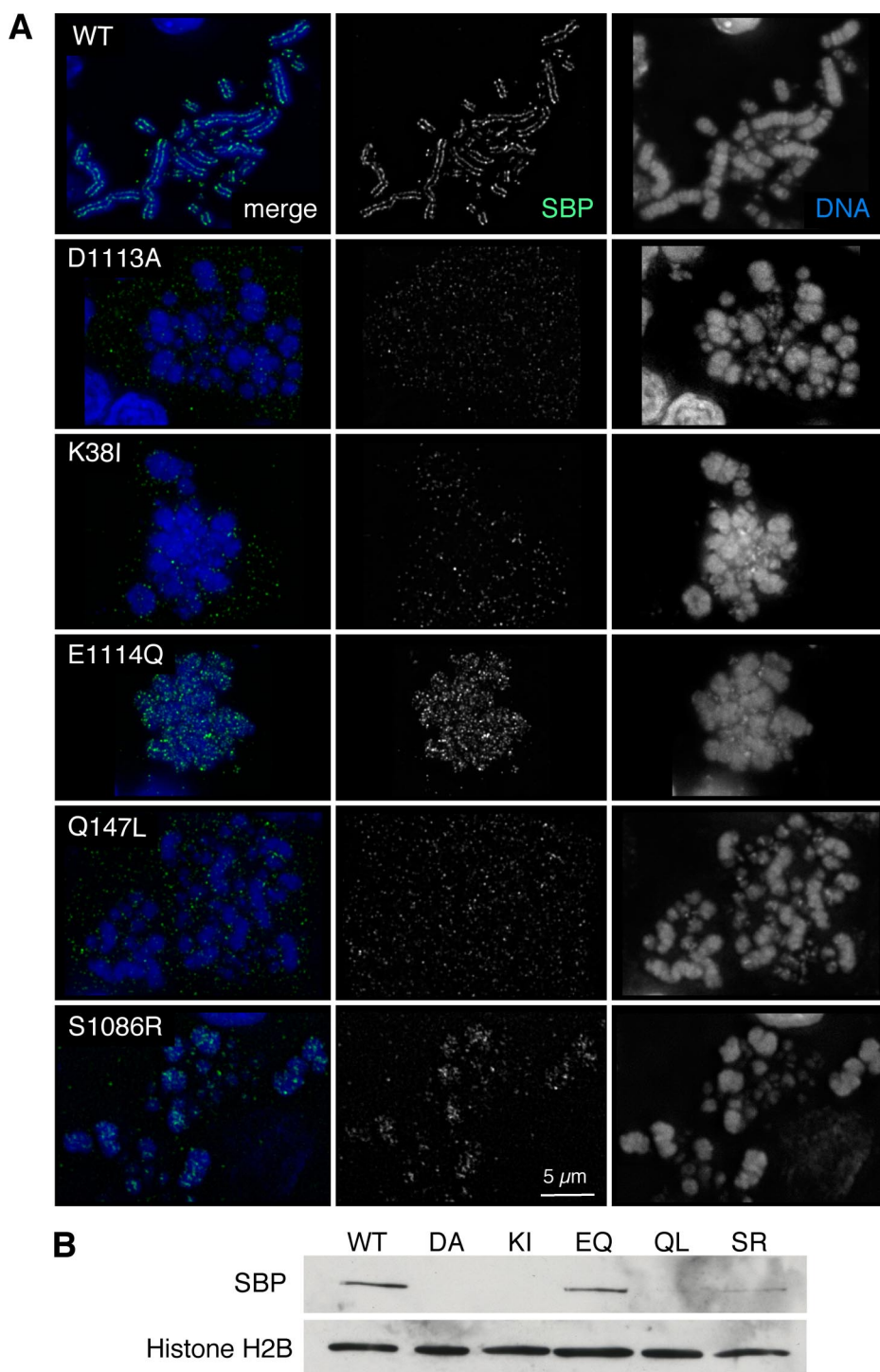


Figure 3. SMC2 ATP binding but not hydrolysis is essential for loading condensin onto mitotic chromosomes. (A) SMC2^{OFF} cells were transiently transfected with WT and SMC2 ATPase mutants tagged with SBP and grown on concanavalin A-coated coverslips. Chromosomes were stained with anti-SBP antibody. Only the SMC2 E1114Q “transition state” ATP hydrolysis mutant and S1086R ATP hydrolysis mutants were able to localize to mitotic chromosomes. Both were unable to restore normal chromosome structure or localize in an axial manner like WT SMC2. The SMC2 ATP binding mutations K38I, D1113A and the Q-loop mutant Q147L prevented condensin loading onto chromosomes. (B) Immunoblotting analysis of chromosomes isolated from stable cell lines expressing SBP-tagged SMC2 wild-type and mutant cells with anti-SBP (top). Anti-histone H2B was used as a loading control (bottom).

condensin complexes containing SMC2 mutations affecting ATP hydrolysis (E1114Q and S1086R) did load onto chromosomes (Figure 3A). The transition state E1114Q mutant seemed to load most strongly onto the chromosomes, whereas some diffuse staining in the cytoplasm of mitotic cells was still seen in cells expressing the S1086R mutant. Importantly, although condensin complexes containing these two mutant forms of SMC2 bound to chromosomes, SMC2 was dispersed throughout the mitotic chromatin rather than showing the normal axial staining, and chromo-

some structure seemed aberrant, reminiscent of SMC2^{OFF} chromosomes after similar treatments.

To confirm that these results were not due to masking of epitopes, we isolated mitotic chromosomes from stable cell lines expressing the SBP-tagged mutants. Blotting using anti-SBP antibody confirmed the results of the immunofluorescence analysis (Figure 3B). SMC2-E1114Q bound to chromosomes at levels comparable with wild type. SMC2-S1086R was also detected on chromosomes, albeit at lower levels. These results suggest that ATP binding might either cause a

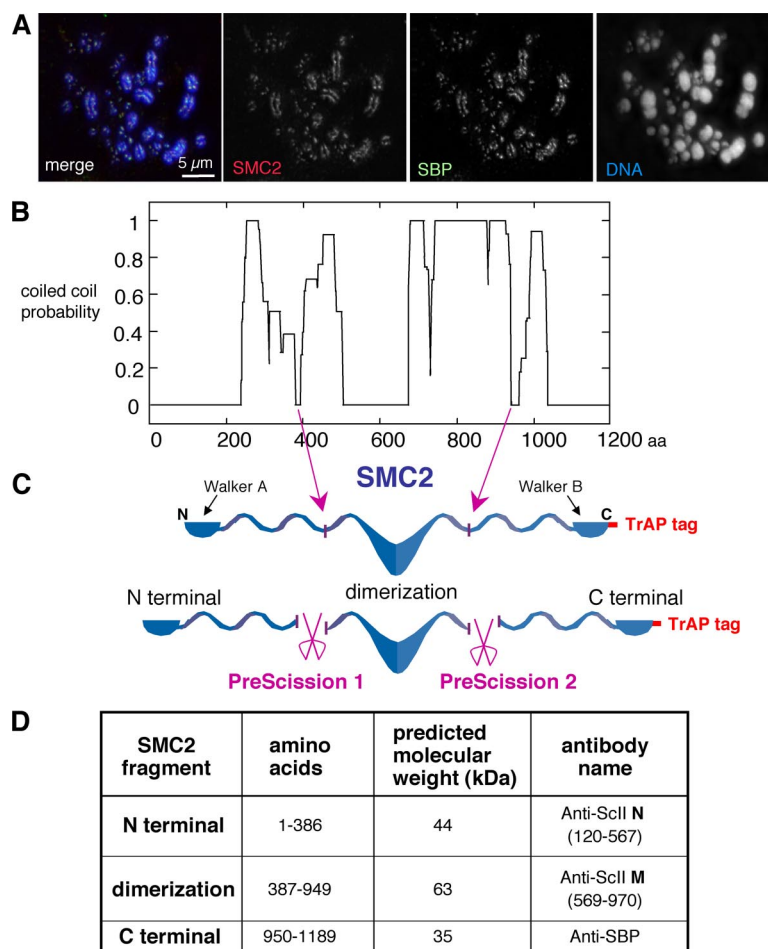


Figure 4. Insertion of PreScission protease sites into SMC2. (A) The cleavable SMC2 is tagged with SBP/S/His and expressed as the only form of SMC2 present in DT40 cells. Costaining with SBP (green) and SMC2 (red) antibodies reveals normal localization and chromosome morphology in the absence of PreScission protease. (B) Coiled-coil probability for the primary sequence of SMC2 *G. gallus* was calculated using PAIRCOIL (Berger *et al.*, 1995). PreScission protease sites were inserted at amino acids 386 and 949 in regions predicted to have 0 probability of a coiled-coil. (C) Schematic depicting the two PreScission protease sites inserted into the coiled-coil regions of SMC2. Cleavage generates three SMC2 fragments. (D) Predicted molecular weight of SMC2 fragments after PreScission protease digestion and fragment-specific antibody used for detection.

conformational change or allow association with another factor necessary to enable condensin to load onto mitotic chromosomes.

Topological Integrity of the SMC2-SMC4 Heterodimer Is Not Required for the Stability of Condensin I and Condensin II Complexes

Studies of cohesin using a tobacco etch virus-cleavable subunit have led to a model in which cohesin functions as a ring that embraces both sister chromatids (Gruber *et al.*, 2003; Ivanov and Nasmyth, 2005). To determine whether condensin also functions as a ring, albeit within rather than between sister chromatids, we constructed a form of SMC2 that could be cleaved by PreScission protease at two balanced sites in the coiled-coil region.

The primary sequence of SMC2 revealed two short stretches of low probability of coiled-coil formation within the arms of SMC2 (Figure 4B). These were chosen as sites for insertion of the eight-amino acid PreScission recognition sequence. Subsequent cleavage would generate three SMC2 fragments (Figure 4, C and D). A triple affinity tag containing S/SBP and His tags was inserted at the C-terminal end of SMC2 to allow affinity purification of the condensin complex containing cleavable SMC2. Remarkably, this cleavable SMC2 construct was able to sustain life and complement SMC2^{OFF} cells. Mitotic chromosome spreads from SMC2^{OFF}SMC2^{PRESCISSION} cells showed normal chromosome morphology and SMC2 staining (Figure 4A). Thus, TrAP-tagged SMC2 with PreScission protease sites inserted

in its arms is fully functional for condensin complex assembly and function in vivo.

To determine the effect of cleavage of the SMC2 arms on the integrity of the condensin complex, complexes containing cleavable condensin were isolated via the TrAP tags and bound to streptavidin beads (Figure 5, B–H). Added PreScission protease efficiently cleaved SMC2 into its middle, C- and N-terminal fragments (Figure 5, B–D), which corresponded to predicted sizes (Figure 4D). After stringent washing, all three fragments remained attached to the beads. The N and C terminal fragments were most resistant to washing; some of the middle fragment was released into washes and supernatant (Figure 5B). This suggests that head-to-head interaction of the SMC proteins may be stronger than interactions involving the dimerization region. Remarkably CAP-H and CAP-D2 binding was virtually identical between control and PreScission digested fractions and only a small fraction of SMC4 was lost (Figure 5, E–G, respectively).

An alternative approach was used to more rigorously test the stability of the complex containing cleaved SMC2 (Figure 5I). Cleavable condensin bound to streptavidin beads was treated with PreScission protease, washed with a buffer containing the ionic detergent deoxycholate, and then subsequently eluted using biotin. The eluted fraction was then rebound to S protein agarose beads, washed as before, and the fraction remaining on the beads was characterized. Silver stain analysis revealed that all three SMC2 fragments remained associated after two purification steps. Mass spec-

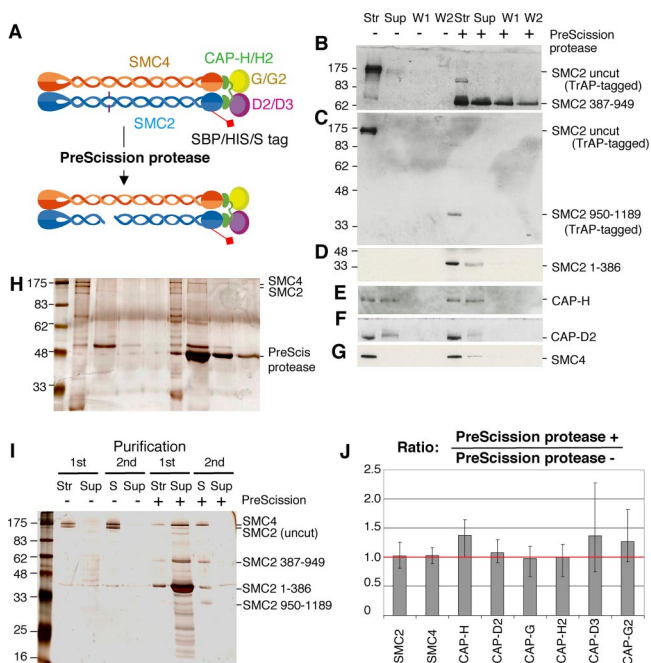


Figure 5. Integrity of the SMC2-SMC4 heterodimer is not required for the stability of condensin I and condensin II complexes. (A) Depiction of the condensin complex as a ring structure where the non-SMC subunits close the ring. Addition of PreScission protease would break this structure. A triple affinity tag (S/SBP/His) at the C terminus of SMC2 allows affinity purification. (B–H) Analysis of pull-downs from condensin isolated from SMC2^{OFF}SMC2^{PRESCISSION} cells. Lysates were bound to streptavidin beads by using the SBP-tag and then PreScission was added. After the supernatant (Sup) was retained, the beads were washed three times (W1, W2, W3 [not shown]), and the sample remaining on the streptavidin beads (Str) was eluted by boiling in sample buffer. Immunoblotting was then performed using an antibody to the middle region of SMC2 (B), the C-terminal using anti-SBP (C), the N-terminal (D), CAP-H (E), CAP-D2 (F), SMC4 (G), and the corresponding samples were silver stained (H). A more stringent approach was next applied using tandem affinity purification technology. (I) Condensin was bound to streptavidin beads, cleaved by addition of PreScission protease, and eluted with biotin after three washes. The C-terminal fragment of SMC2 was then rebound to S-protein agarose beads and eluted by boiling in sample buffer. The biotin elution from Str, Sup, and S protein agarose (S) elution were silver stained. (J) The ratio of peptide number for condensin subunits from the streptavidin bead elutions (Str) and S protein agarose elutions (S) plus and minus PreScission protease showed no significant loss of either condensin I or II subunits. Data were taken from four experiments. Even after two purification steps (S elution), there is no significant loss in condensin subunits (also see Supplemental Figure S2).

trometry confirmed that both condensin I and II complexes were present in approximately equivalent amounts and that there was no significant loss of any of the SMC2 fragments (Figure 5J and Supplemental Figure S2), suggesting both strong intermolecular and also intramolecular interactions between the coiled-coil domains of SMC2.

Condensin Remains Tightly Bound to Chromatin After Cleavage of SMC2

Analogous studies of yeast cohesin found that cleavage of the SMC3 cohesin subunit was sufficient to release the complex from chromatin (Gruber *et al.*, 2003). We therefore wished to determine whether condensin could also be released upon cleavage of its SMC2 subunit. However isolated chromosomes digested with PreScission protease showed

only slight reductions in the levels of associated SMC2, SMC4, and CAP-H (Figure 6, B–D), suggesting the complex is remarkably stable once assembled into mitotic chromatin. Immunofluorescence of purified chromosomes treated with PreScission protease also showed no obvious differences in staining using the scaffold marker KIF4A and the SBP antibody (Figure 6G). Chromosomes purified and treated with PreScission protease retained similar architecture, and they seemed to have axial staining characteristics similar to mitotic chromosomes. This appearance is distinct from chromosomes lacking SMC2, in which scaffold markers become diffuse and chromosome architecture becomes abnormal (Hudson *et al.*, 2003) (Supplemental Figure S3).

Previous analysis of scaffold fractions from chromosomes lacking SMC2 showed that the presence of condensin is essential for scaffold integrity (Hudson *et al.*, 2003). Known scaffold components were absent from chromosome scaffolds isolated from SMC2^{OFF} cells as revealed by immunoblotting and the total recovery of Coomassie-stained proteins in the scaffold fractions were also significantly reduced. Although condensin components seemed normal in the scaffold fraction after condensin was cleaved by PreScission protease, there was a substantial (>50%) increase in the solubilization of the major chromosome scaffold component DNA Topo II α (Figure 6, E and H, and Supplemental Figure S4). The change in topoisomerase II α solubility was selective, as no significant change was seen in the level of scaffold-associated kinesin KIF4A (Figure 6, F and H, and Supplemental Figure S4). The overall protein content in the supernatant fraction of scaffolds for chromosomes treated with PreScission was also significantly increased (Figure 6A, lane 6). Thus, although the scaffold fraction is retained when condensin is cleaved, the overall architecture is compromised leading to destabilization of individual scaffold components.

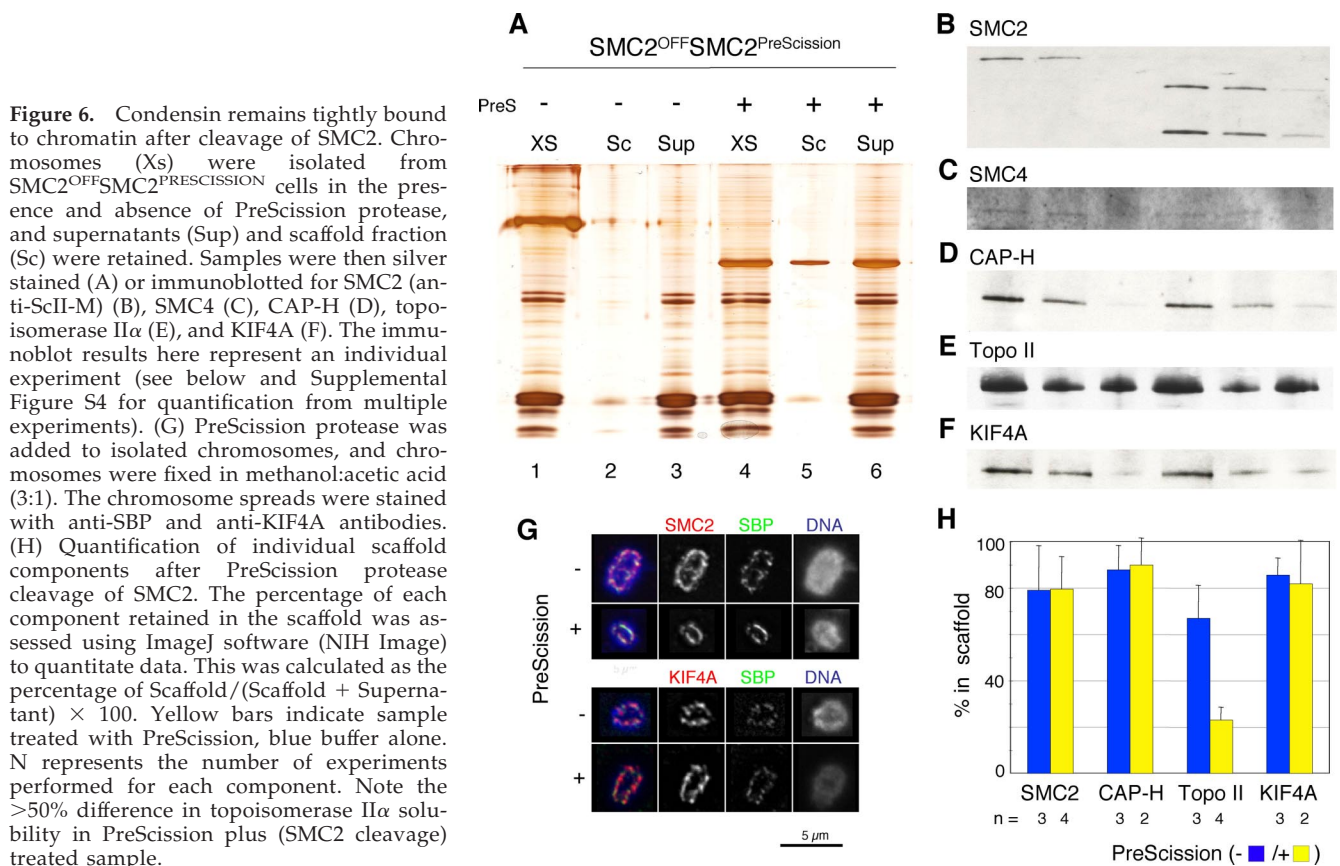
DISCUSSION

Cohesin and condensin are the two most widely studied nonhistone protein complexes with important roles in mitotic chromosome structure and behavior. The present study serves to highlight that even though these two complexes are superficially similar (e.g., both are built around SMC protein heterodimers), the role played by ATP in the function of the two complexes is apparently distinct.

Mechanistic Differences Between Cohesin and Condensin ATPase Function

Our study shows ATP binding and hydrolysis by SMC2 are not required for the assembly of the condensin holocomplex. A separate study using baculovirus purified condensin I also found that ATPase mutations in either SMC2 or SMC4 failed to affect the formation of the holocomplex *in vitro* (Onn *et al.*, 2007). In contrast, ATP binding in the cohesin subunit SMC1 but not SMC3 is essential for interactions with Scc1 and therefore for assembly of the cohesin complex (Arumugam *et al.*, 2003; Weitzer *et al.*, 2003).

We also report the first functional analysis of the conserved Q-loop found in all SMC proteins. This motif has been proposed to bind water and magnesium and to undergo a conformational change upon substrate binding (Hopfner *et al.*, 2000; Hopfner and Tainer, 2003). It has been suggested that this acts as a “lever arm” to transduce ATP binding and hydrolysis into physical action by the SMC protein (Hopfner and Tainer, 2003). We found that SMC2 Q-loop mutant Q147L has no effect on the formation of the condensin holocomplex, but abolished its association with



chromosomes. It remains to be determined whether this Q-loop mutant has impaired ATPase function or acts downstream of ATP to disrupt a conformational change required for condensin loading.

ATP-binding, but not hydrolysis is required for the association of condensin with mitotic chromosomes in vivo. Despite the fact that SMC2 mutants D1113A and K38I, which are predicted to block ATP binding, are able to participate in formation of the condensin complex, complexes containing these mutations failed to associate with chromosomes. In contrast, the SMC2-E1114Q mutant, which is predicted to slow the rate of ATP hydrolysis, bound to chromosomes at levels comparable with wild type. The SMC2-S1086R mutant bound chromosomes less well, possibly reflecting its ability to bind, but not to hydrolyze, ATP. These results are consistent with those of an in vitro study of *Bacillus subtilis* SMC homodimers, in which transition state mutants (analogous to SMC2-E1114Q) allowed detectable DNA binding (Hirano and Hirano, 2004). In contrast, cohesin complexes with the analogous ATP hydrolysis mutation in either SMC1 or SMC3 fail to load onto chromatin (Arumugam *et al.*, 2003).

Our in vivo studies of condensin function are consistent with recent in vitro studies from the Hirano laboratory, which showed that purified SMC2 undergoes a conformational shift in the presence of ATP, leading to the suggestion that ATP binding might open the hinge region (Onn *et al.*, 2007). Recent studies of cohesin have shown that loading of the complex onto chromatin is caused by transient opening of the hinge domain, and it was hypothesized that this conformational change could be the result of either ATP binding or hydrolysis (Gruber *et al.*, 2006). Thus, it seems that ATPase activity within the head domain could relay the

conformational changes required to open the hinge region for SMC proteins (Figure 7).

Regardless of the mechanism, cells expressing the mutated ATPase domains in SMC2 are unable to form functional condensin complex. Although the results reported here represent a first attempt using a genetic approach to understanding the role of condensin ATPase function in vivo, further work is required for a definitive characterization of the ATPase cycle in purified condensin.

Cleavage of SMC2 Does Not Alter Condensin Association or Binding to Chromosomes in Vitro

One key to understanding the function of condensin is to ascertain whether the complex forms a closed ring structure and traps DNA in a manner analogous to that proposed for cohesin (Ivanov and Nasmyth, 2005). Electron microscopy studies reveal that the cohesin arms seem to form an open loop, and they are thus topologically in a position to encircle DNA, whereas condensin predominately forms a "lollipop" like structure with the arms tightly apposed to one another (Anderson *et al.*, 2002). Our observations that the condensin complex remains largely intact despite the cleavage of SMC2 are consistent with the notion that condensin arms are apposed in a lollipop structure. Our results thus support the notion that condensin acts via a mechanism distinct from cohesin.

By analogy to experiments with the cohesin subunit SMC3 (Gruber *et al.*, 2003), cleavage sites were chosen that would break SMC2 in regions of lowered propensity for coiled-coil formation and therefore would not interfere with the structure of the complex. However, we cannot exclude the possibility that interactions between SMC2 helices (or with

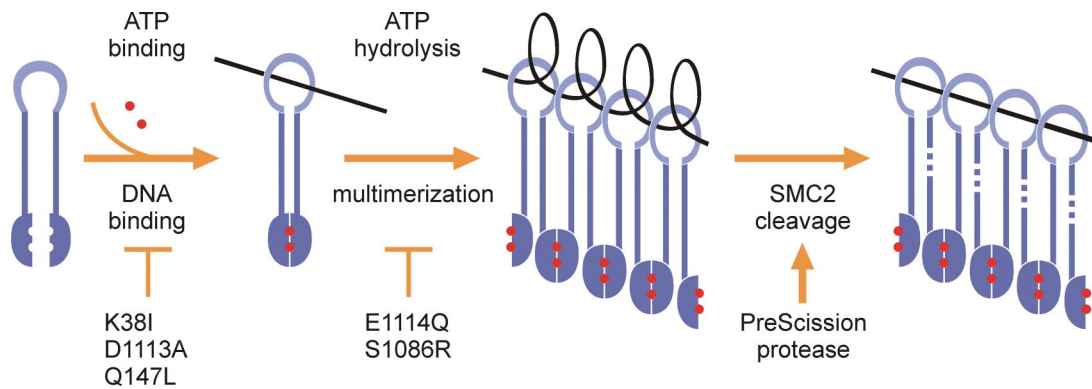


Figure 7. Hypothetical working model for condensin binding to chromosomes showing ATP binding producing a conformational change that allows DNA entry into an altered hinge region. Mutations blocking ATP binding (K38I, D1113A) or allosteric change (Q147L) inhibit condensin loading onto chromosomes. ATP hydrolysis results in the disengagement of the SMC2 and SMC4 head domains allowing intermolecular multimerization between condensins and formation of higher order structures. Mutations affecting ATP hydrolysis (S1086R, E1114Q) allow condensin to associate with chromatin but may affect the multimerization properties of the complex. In contrast to cohesin, condensin remains associated with chromatin after breakage of a SMC subunit most likely due to strong intermolecular interactions between the coiled-coil regions of SMC2 and SMC4.

SMC4) in the coiled-coil might be retained even after protease cleavage. After *in vitro* cleavage of SMC2 by PreScission protease, a significant portion of the middle hinge/dimerization region could be substantially solubilized (Figure 5B), consistent with cleavage rather than simply “nicking” the SMC2 coiled-coil, whereas the C and N domains remain tightly associated (Figure 5, C and D). In the previous study of cohesin, when cleavable SMC3 was expressed and cleaved *in vitro* on beads, approximately half of the dimerization domain was released, suggesting comparable cleavage of SMC proteins between the two systems (Gruber *et al.*, 2003). However, given the predicted lollipop conformation for the condensin holocomplex, we cannot say whether cleavages within the SMC2 coiled-coil open the complex entirely when the complex is bound to chromosomes.

Cleavage of SMC3 releases the cohesin complex from chromatin and can initiate the onset of sister chromatid separation, even though it does not alter the interactions between SMC3, SMC1, or Scc1 (Gruber *et al.*, 2003). When SMC2 in purified condensin is cleaved by PreScission protease, the complex seems to remain intact without any significant loss of either the condensin I or II non-SMC subunits. This was true even under stringent tandem purification conditions with multiple washes in a buffer that included the ionic detergent deoxycholate. Furthermore, when isolated chromosomes were treated with PreScission protease, SMC2 remained concentrated along the chromatid axes despite being quantitatively cleaved. Therefore, condensin complex stability and association with mitotic chromosomes is not dependent upon the integrity of the SMC2 heterodimer. In contrast, the chromosome association of DNA topoisomerase II α was specifically altered after SMC2 cleavage. This demonstrates that the PreScission cleavage of SMC2 did indeed alter condensin structure or function, and it suggests a close association of Topo II α with the condensin complex in chromosomes.

Cleavage of the SMC2 coils might be expected to release the complex from chromatin if condensin were to bind DNA by an “embrace” model as proposed for cohesin. However, the failure to release the complex from chromosomes after SMC2 scission suggests that chromosome association by condensin may not solely require topological closure of the complex. Thus, the SMC arms of condensin might transmit

conformational changes that enable loading or unloading of the complex.

To date the only direct visualization of condensin associated with DNA was provided by atomic force microscopy of the purified fission yeast complex (Yoshimura *et al.*, 2002). The work showed condensin as sitting on DNA with its hinge but not topologically embracing DNA, and in some instances with the heads bending down to the DNA. It is possible, however, these images represent condensin trapped in a preloading state because of the limited biochemical activity of the preparation or absence of loading factors (Uhlmann and Hopfner, 2006).

The way in which condensin interacts with DNA therefore remains an open question. Our *in vivo* data have demonstrated the importance of the ATPase cycle of condensin in regulating this process. Together, our work and the work performed by others have served to highlight a SMC paradox in which remarkably similar proteins that form highly analogous complexes seem to function by distinct mechanisms.

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